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Mycotoxin Synergism

E. B. LILLEHOJ AND A. CIEGLER

Northern Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604

INTRODUCTION

Ever since the carcinogenic metabolites of *Aspergillus flavus* in peanuts were identified, attention has been directed to the broad implications of mycotoxin hazards. In the post-aflatoxin era, mycotoxin studies generally were devoted to: (i) occurrence and characterization of specific toxins, (ii) examination of factors that contribute to the elaboration of the toxic substances, and (iii) description of the effects of particular fungal metabolites on microbes, plants, and animals (23, 46). Although the presence of a toxin in foods and feeds represents an obvious disease potential, there is more concern growing over the health hazard associated with the simultaneous occurrence of two or more toxins. Diverse fungal products can be expected to accumulate in a substrate either through simultaneous elaboration of several compounds by a single strain or by sequential development of several species and associated toxin production.

Mycotoxicologists are particularly conscious of toxic interactions in which the cooperative effect of two or more substances elicits a total effect greater than the sum of the activities of individual agents; this combined response is called a toxic synergism. Aggregate toxicities in test animals depend on a number of variables, including species, sex, age, nutrition, disease, test procedures, and environmental conditions (temperature, light, and humidity). In aflatoxicosis, young animals are more susceptible than older ones, males more than females, and both ducklings and trout are significantly more sensitive than mice (23, 46). Within the inherent constraints, several techniques have been developed and adapted to the determination of mycotoxin synergism in test animals. These involve growth rates, organ function, acute toxicity, tissue histology, cellular function, blood chemistry, immunological response, and tumor induction.

Particularly useful in measuring mycotoxin synergism is the initiator-promoter procedure employed in the mouse skin tumor test. This unique technique provides a two-step process in which single applications of an initiator substance, e.g., 9,10-dimethyl-1,2-benzanthracene (DMBA), and subsequent treatments with promoting substance, e.g., croton oil, produce tumors on sensitive mouse strains (8). Similar dose levels of either initiator or promoter alone are ineffective in causing skin tumors.

MYCOTOXIN-MYCOTOXIN SYNERGISM

The progression of fungal flora on agricultural commodities generally includes species of *Fusarium*, *Penicillium*, and *Aspergillus* (13). *Fusarium* mycotoxicoses have been attributed to the presence of metabolites of the trichothecene group, e.g., T-2 toxin and diacetoxyscirpenol (4). Characteristically, trichothecenes elicit a severe irritation and inflammation on contact with human skin and with test animals. In a study of synergism between aflatoxin B_1 and T-2 in acute toxicity, the LD_{50} values of toxin pairs exhibited a distinct increase in toxicity (47; Fig. 1). The LD_{50}s plotted by Hewlett's (39) method are presented in Fig. 1; the decrease in values of the toxin pairs represents a synergistic response. In addition to acute toxicities, the interaction among aflatoxin B_1, T-2, and diacetoxyscirpenol was examined by the mouse skin tumor test (47). B_1 was a tumor initiator; application of a single 25-μg dose with subsequent croton oil promotion caused extensive tumor development. Trichothecenes did not serve as initiating agents but did exhibit weak promoting activities on the skin of test mice initiated with DMBA (47, 55). The trichothecene-induced skin irritation clearly did not relate effectively to enhanced skin tumor formation.

Mycotoxicoses of domestic animals have been attributed to ingestion of feedstuffs simultaneously contaminated with *A. flavus* and *Penicillium rubrum* (10). Subsequent studies identified and characterized two structurally similar toxins from *P. rubrum*, named rubratoxins A and B (117); the substances are not carcinogenic but are quite toxic; e.g., LD_{50} values below 1 mg/kg have been observed in several animal species (61). Rubratoxins appear to be primarily hepatoxins (61, 122). Studies on the synergistic activity of rubratoxin B and aflatoxin B_1 in rats provided evidence that the lethality of rubratoxin B (25

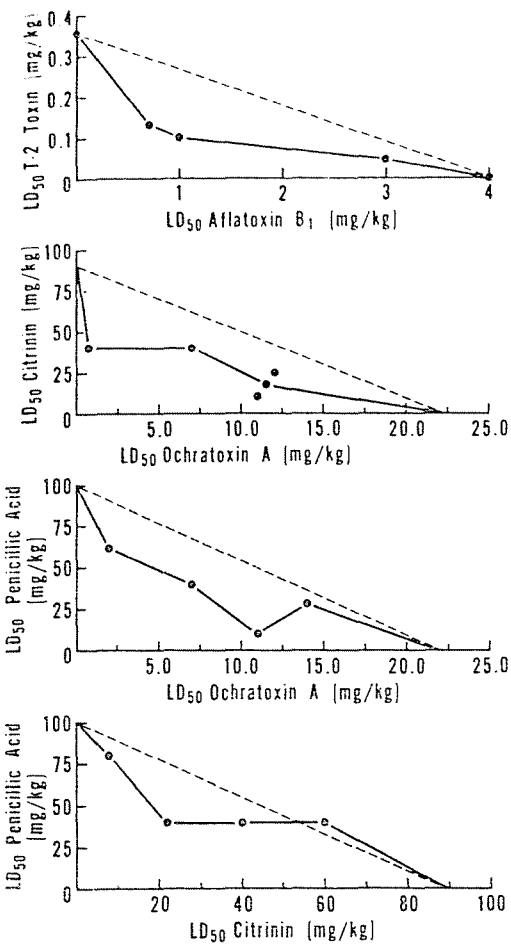

FIG. 1. *Isobologram for two mycotoxins injected intraperitoneally into white mice. Dashed line represents expected LD_{50}s, assuming additive response; solid line, observed LD_{50}s for various combinations of the two toxins (47; Sansing, Lillehoj, and Detroy, in preparation).*

mg/kg, 3 times/week, for 5 weeks) was expressed only if the animals were fed a sublethal level of aflatoxin B_1 (0.2 ppm) during the trial period (122). However, rubratoxin B did not appear to potentiate the carcinogenicity of aflatoxin B_1. In an extensive examination of toxic interaction between dietary aflatoxin (2.5 ppm) and rubratoxin (500 ppm), Wyatt et al. (123) observed effects in broiler chicks. After 3 weeks on the test ration, the chicks displayed no synergistic toxic effects in growth rate, relative organ weights, hemoglobin, serum cholesterol, and total serum lipid. In a fascinating study with guinea pigs, Richard et al. (90) observed the effects of rubratoxin alone and in combination with aflatoxin on several experimental variables. Toxin pairs administered orally for 3 weeks increased lethality and substantiated earlier reports of synergism of the two substances in acute toxicity tests. Rubratoxin alone reduced complement activity at 6 mg/day for 3 weeks, whereas at 4 mg/day no reduction was detected; addition of 0.01 mg of aflatoxin B_1 to the latter treatment, however, significantly decreased activity. Levels as low as 0.03 mg of B_1/day decreased complement. Since mixed mycotoxin effects involving rubratoxin have been associated with a hemorrhagic syndrome (6), Richard et al. (90) examined prothrombin time in treated guinea pigs. Rubratoxin alone caused a slight increase in the prothrombin time; this was significantly enhanced after treatment with toxin combinations. Rubratoxin-aflatoxin combinations also reduced growth in both guinea pigs (90) and infant rats (J. A. Cain, A. W. Hayes, and B. G. Moore, in preparation). Richard et al. (89) also investigated the effect of ochratoxin in combination with aflatoxin on complement activity, serum proteins, and antibody response in guinea pigs. No synergistic toxic response was observed, nor was there significant interaction between aflatoxin and antigen in lowering serum albumin levels.

An intriguing consideration of potential mycotoxin synergisms is the interaction between two or more structurally similar metabolites that are produced by a fungus simultaneously. Routinely, *A. flavus* produces aflatoxins B_1 and B_2, and *A. parasiticus* synthesizes aflatoxins B_1, B_2, G_1, and G_2 (23, 38, 46). Toxin-producing strains of both species also elaborate aflatoxin M (23). Acute toxicity results indicate that the order of potency of individual aflatoxins is $B_1 = M_1 > G_1 > B_2 > G_2$ (121). Aflatoxin G_1 is hepatocarcinogenic, but higher doses of G_1 than of B_1 are required to initiate tumors. However, G_1 is more effective as an inducer of renal tumors than B_1 (23, 121). Aflatoxin M_1 is approximately as carcinogenic in trout as B_1. Ayres et al. (3) reported the carcinogenic interaction of B_1 and B_2. Hepatoma incidence in trout after 12 months on a toxin-containing ration was as follows: 4 ppb of B_1, 25%; 8 ppb of B_1, 70%; 20 ppb of B_2, 5%; and 4 ppb of B_1 and 4 ppb of B_2, 43%. There is a distinct synergism between B_1 and B_2 in inducing trout liver tumors.

Reports of the natural contamination of grains by citrinin, ochratoxin, or penicillic acid have stimulated work on the toxic interaction of these mycotoxins (42, 102, 115). The simultaneous occurrence of ochratoxin and citrinin in naturally contaminated commodities has also been observed (42, 105). Fungal

strains have been isolated that concomitantly produce either ochratoxin and citrinin (15, 42, 105) or ochratoxin and penicillic acid (14). In addition, isolates of the fungus responsible for the contamination of grains with ochratoxin, *Penicillium viridicatum*, have also produced citrinin and penicillic acid (102). In a study of the interaction effects of ochratoxin and penicillic acid in mice, mixtures of the two mycotoxins produced a synergistic lethal response (Fig. 1; 48; G. A. Sansing, E. B. Lillehoj, and R. W. Detroy, in preparation). However, neither of the toxins functioned as initiating or promoting agents in the mouse skin tumor test.

When Ciegler, Mintzlaff, and Leistner (personal communication) administered ochratoxin plus penicillic acid to rats and quail by intubation, weight gains were lower and lethality was higher than anticipated from an additive toxic response (Tables 1 and 2). Since Ciegler et al. (16) had observed that strains of *Penicillium* were capable of producing both citrinin and patulin, this mycotoxin pair was also examined. Simultaneous addition of the two toxins to quail yielded results similar to those observed with ochratoxin-penicillic acid (Table 2); all the birds given the toxin pairs were dead within 24 h after treatment, whereas none of the quail treated with single toxins died dur[ing] the same period. In the chick embryo, individual mycotoxins increased lethality, but th[ere] was no significant synergism by toxin pairs [of] ochratoxin-penicillic acid and patulin-citr[inin] (Table 3).

In studies of the toxic interaction of ochr[a]toxin, citrinin, and penicillic acid by Sansing et al. (in preparation), combinations of citrinin ochratoxin and citrinin-penicillic acid caused synergistic lethal response in mice. This response was expanded by examining the effects of toxin pairs on nucleic acid metabolism in liver and kidneys of mice. Generally, toxin combinations initiated effects similar to the independent functions of each mycotoxin. Although penicillic acid alone stimulated ribonucleic acid (RNA) synthesis in liver, combinations with ochratoxin A or citrinin inhibited accumulation of the nucleic acid.

In infant rats, simultaneous treatment with ochratoxin and rubratoxin gave a synergistic response; the LD_{50} of ochratoxin (3.9 mg/kg) was reduced about 16 times to 0.24 mg/kg in the presence of 5 mg of rubratoxin/kg (LD_{50} = 6.38 mg/kg; Cain, Hayes, and Moore, in preparation).

Zwicker and Carlton (124) examined the interaction of the carcinogenic mycotoxin

TABLE 1. *Response of rats to doses of ochratoxin and penicillic acid*[a]

Treatment	Mean wt (g)				25-day gain (g)	Deaths (%)
	Day 0	Day 8	Day 19	Day 25		
Control	90	131	197	215	125	0
Ochratoxin	76	85	112	125	49	0
Penicillic acid	76	123	148	168	92	0
Ochratoxin + penicillic acid	83	84	—[b]	—	0	100

[a] From A. Ciegler, H. J. Mintzlaff, and L. Leistner (personal communications). Five rats were treated per dose level. Toxins were intubated in 2-ml doses, three times a week, at the following dose levels: ochratoxin, 1.6 mg; penicillic acid, 44 mg. Toxins were dissolved in 0.1 M $NaHCO_3$.

[b] All rats died by day 10 of the trial.

TABLE 2. *Response of Japanese quail (Coturnix japonica) to doses of ochratoxin and penicillic acid*[a]

Treatment	Mean wt (g)				21-day gain (g)	Deaths (%)
	Day 0	Day 7	Day 14	Day 21		
Control	44	62	73	90	46	0
Ochratoxin	46	54	49	54	8	33
Penicillic acid	47	56	61	84	37	13
Ochratoxin + penicillic acid	46	47	40	—[b]	—	100

[a] From A. Ciegler, H. J. Mintzlaff, and L. Leistner (personal communications). Fifteen quail were treated per dose level. Toxins were intubated in 1-ml doses, three times a week, at the following dose levels: ochratoxin, 0.75 mg; penicillic acid, 5 mg. Toxins were dissolved in 0.1 M $NaHCO_3$.

[b] All test quail died by day 15 of the trial.

TABLE 3. *Toxicity of mycotoxin combinations to the chick embryo*[a]

Treatment	Deaths/total no. of embryos		Percent deaths (mean)
	Test 1	Test 2	
Control	6/62	8/75	10.2
Ochratoxin	24/80	21/75	29.0
Penicillic acid	18/78	16/75	22.2
Ochratoxin + penicillic acid	41/80	41/75	52.9
Control	15/80	7/77	14.0
Patulin	45/80	38/77	52.8
Citrinin	20/80	15/77	22.3
Patulin + citrinin	58/80	69/77	80.9

[a] From A. Ciegler, H. J. Mintzlaff, and L. Leistner (personal communication). All toxins were injected into the air sac in 0.1-ml doses of 0.1 M $NaHCO_3$ at the following dose levels: ochratoxin, 5 μg; penicillic acid, 150 μg; patulin, 4.0 μg; citrinin, 50 μg. Controls received 0.1 ml of 0.1 M $NaHCO_3$.

sterigmatocystin (85) and a diet containing rice infected with *P. viridicatum*. In mice fed a diet containing 5 ppm of the mycotoxin, the incidence of plumonary tumors was 73% higher than in controls and 16% higher in test animals on the fungus-contaminated diet. However, addition of 5 ppm of sterigmatocystin to the *P. viridicatum*-infected ration induced tumors in only 54% of the animals. There was no evidence to verify any synergistic activity between the two test materials.

Almost all mycotoxin studies have considered the toxicity of small-molecular-weight metabolites. However, proteinaceous macromolecular fungal endotoxins have been described (116). In addition, Sansing et al. (96) identified the toxic properties of another macromolecular constituent of fungal cells, the virus-like particles (VLP) and attendant double-stranded RNA (ds-RNA) from *P. stoloniferum*. The LD_{50} in mice for VLP was 240 mg/kg and for ds-RNA was 34 mg/kg. Combining ds-RNA with a series of mycotoxins produced no synergistic lethal effect in mice. However, administering sublethal levels of actinomycin D or cycloheximide dramatically enhanced the toxicity of fungal VLP and ds-RNA.

In a preliminary test, Ciegler (personal communication) found that rabbits responded more rapidly and to a greater extent when dosed per os with a crude extract of tremorgenic toxins (largely penitrem A) produced by *P. cyclopium* than when dosed with larger quantities of penitrem A alone. Wilson et al. (120) also noted that administration of crude extracts of tremorgens caused a greater toxic response in rats than equivalent doses of pure toxin. The factor mediating the potentiation of penitrem A toxicity has not yet been elucidated.

MYCOTOXIN-NONMYCOTOXIN SYNERGISM

Diets that led to hepatoma epizootics in trout contained cottonseed meal. Constituents of the cottonseed meal were associated with the tumor induction (23, 46, 106). When aflatoxin was discovered in the rations, it was implicated as the primary tumor-inducing factor, but purified rations with added aflatoxin did not cause the level of tumor incidence observed when the trout were fed aflatoxin in untreated meals (43). Since the cyclopropenoid fatty acids (CPFA) malvalic and sterculic acids occur in cottonseed, these substances were tested for carcinogenic synergism (43, 104). A control diet containing 4 ppb of B_1 produced a 50% tumor incidence in trout after 1 year; addition of 56 ppm of CPFA to the toxic diet induced a 100% tumor occurrence. *Sterculia foetida* oil (49% sterculic and 7% malvalic acids) was the source of CPFA (43). Increase of the CPFA level to 220 ppm increased the hepatoma incidence sixfold in the presence of 4 ppb of the aflatoxin (43). A 4-ppb level of aflatoxin M_1 induced a 13% incidence of hepatoma in trout after 1 year; addition of 100 ppm of CPFA increased the occurrence to 70% (105). Addition of another cottonseed constituent, gossypol, at 250 ppm or of 3-methylcoumarin at 50 ppb doubled the hepatoma incidence in trout on a diet containing 4 ppb of aflatoxin B_1 (104). Without aflatoxin, the two synergists did not elicit tumor formation.

Lee et al. (44) investigated the toxic interaction between CPFA and aflatoxin in rats. After 18 months on a ration containing 18.4 ppb of aflatoxin, 59% of the animals had developed tumors, whereas addition of 220 ppm of CPFA increased the tumor incidence to

70%. In a subsequent study (71), hepatoma incidence in rats increased from 41 to 61% when CPFA were added to a diet containing 100 ppb of aflatoxin B_1. A comparison between trout and rat shows that CPFA are more effective synergists in trout.

NUTRITIONAL MODIFICATION OF MYCOTOXIN EFFECTS

Several laboratories have examined intensively the influence that nutritional status of test animals has on their susceptibility to mycotoxicoses. Newberne et al. (70) observed that addition of lysine (0.8%) and arginine (1.0%) to a basal diet sensitized ducklings to the acute toxicity of aflatoxin. They postulated that increased transaminase and deaminase activity by the liver could be responsible for the increased lethality. Foy et al. (25) emphasized the importance of diet in potentiating the effect of toxic insult in animals. They demonstrated a similarity in the response of baboons to pyridoxine deficiency and aflatoxin-induced toxicity and proposed that the two functioned synergistically in eliciting liver damage. Since reduction in pyridoxine probably impairs the capacity of cells to carry out trans- or deaminations, loading these activities may enhance the toxic effects of aflatoxin.

Madhaven et al. (54) examined the effects of aflatoxin by feeding monkeys a diet containing 4 or 20% protein. All the test animals given 100 μg of aflatoxin/day on the low-protein diet died within 30 days with characteristic liver lesions, whereas animals on the high-protein ration were protected. In similar studies with weanling rats, the animals on low-protein diets were sensitized to the acute toxicity of aflatoxin, but they exhibited a higher incidence of liver tumors than animals on the 20% protein ration (53). On the other hand, Newberne et al. (64), as well as Newberne and Wogan (69), observed that rats treated with 375 μg of aflatoxin B_1 on a 9% protein diet had a higher incidence of tumors in a shorter time than rats on a 22% protein ration. Marcos and Lebshtein (56) studied the effect of aflatoxin on chickens being fed rations containing four different levels of protein. They observed that aflatoxicosis was more severe at the lower protein concentrations and proposed that protein malnutrition predisposed the birds to the toxic effects of the mycotoxin. Smith et al. (107) reported that a ration containing 30% protein gave broiler chickens essentially complete protection against a 5-ppm level of aflatoxin.

Newberne et al. (64) investigated the effects on rats of simultaneous feeding with ethionine and aflatoxin. The amino acid at 0.2% of the diet acted as a carcinogenic synergist with 0.1 ppm of aflatoxin B_1. On the other hand, simultaneous feeding of urethane, a known carcinogen, at 0.1 to 0.6% of the diet with 0.4 to 1 ppm of B_1 restricted tumor development (69). Other substances (phenobarbitone, DDT) also exhibit a protective effect in aflatoxin-mediated toxicities (31, 50, 51).

The association of fatty liver and cirrhosis with liver toxins stimulated investigations on how aflatoxin would affect rats fed a lipotropic-deficient diet. Newberne et al. (64, 67) selected a low-lipotropic diet containing a basal 20% protein ration with 0.1% DL-methionine and 0.1% choline chloride, whereas the lipotrope-supplemented ration contained the same protein constituent with 0.6% DL-methionine, 0.6% choline chloride, and 50 μg of vitamin B_{12}/kg. During the first 2 weeks, 240 μg of aflatoxin B_1 was administered to each test rat. After 1 year on the low-lipotropic diet, 5 of 17 animals developed liver carcinoma, and the remainder had histological changes indicative of preneoplastic changes. Liver carcinoma was not found in the animals on the high-lipotropic diet treated with identical levels of aflatoxin. Subsequent studies (92-94) showed that lipotropic deficiency protected rats against lethal doses of aflatoxin but increased the susceptibility to aflatoxin-induced liver tumors.

The aggregate response to the interaction of light, dietary constituents, and toxins has been investigated. Photosensitization of animals and humans is a serious economic problem in areas where ingestion of photosensitizing substances leads to disease characterized by skin necrosis (76). Sporodesmin, a product of *Pithomyces chartarum*, is an extensively studied photosensitizing mycotoxin; the disease associated with it is called facial eczema and occurs principally in sheep and cattle (88, 113). Sporodesmin, a hepatoxin, produces severe occlusive damage to the bile duct system and, subsequently, causes photosensitization due to a failure of the damaged liver to remove phylloerythrin from the blood. A phototoxin syndrome has also been identified in mice after ingestion of rice cultures infected with *P. viridicatum* (9); this phototoxicity was also attributed to liver damage. Newberne et al. (63) examined the toxic interaction of light, vitamins, and aflatoxin in rats. Carotene and riboflavine were selected for testing, since these substances are photoreceptive agents. Aflatoxin absorbs light at 363 nm, but the photo-

chemistry of the toxin has not been extensively studied (23, 46). Rats exposed for 2 h to light after toxin treatment exhibited enhanced susceptibility to aflatoxin; light plus riboflavine synergistically increased the aflatoxin-mediated toxicity, but carotene provided protection from the light-toxin interaction. A similar aflatoxin-mediated phototoxicity has been observed in cultures of *Paramecium* (109).

Reddy et al. (87) recorded the effect on rats of a dietary deficiency of vitamin A. Males on the vitamin-deficient ration exhibited a distinct increase in the susceptibility to liver damage and lethality expressed by a single dose of aflatoxin. Newberne and Rodgers (66) studied the effects of vitamin A on tumor induction in rats. Aflatoxin-induced liver carcinomas were observed at a frequency anticipated from earlier studies without significant vitamin A effects. However, 11 of 260 animals exposed to aflatoxin developed colon carcinomas, generally in association with the low levels of dietary vitamin A. In related work with vitamins and aflatoxin-induced disease, Hamilton and Garlich (35) found that supplementing rations with choline, inositol, vitamin B_{12}, and vitamin E did not reverse the fatty liver syndrome in chickens induced by aflatoxin.

Pyrrolizidine alkaloids are hepatoxins naturally occurring in several plant species (100). Studies conducted in 1972 showed that the alkaloids are carcinogenic in rats (111). After an examination of the interaction of lasiocarpine, a pyrrolizidine alkaloid, with aflatoxin, Reddy and Svoboda (86) observed that the alkaloid did not inhibit aflatoxin-induced formation of liver tumors but that the toxin pair initiated a pathogenic pattern which was different from the one elicited by aflatoxin alone.

Dietary lipids have been described in terms of their function in aflatoxicosis (121). Particularly interesting studies by Hamilton and his colleagues have shown that increasing the lipid component of a broiler chicken ration from 2 to 16% decreases the toxicity of dietary aflatoxin (5 ppm) to control levels (107). They proposed that a high-lipid diet could prevent absorption from the gastrointestinal tract. Subsequently, a similar sparing effect of high-lipid diets on aflatoxin-induced lethality was observed in turkey rations (37).

Another aspect of mycotoxin-mediated toxicity and interaction with the environment is the increased stress on animals ingesting the toxins; generally, this stress is reflected in reduction of growth rate, inefficient feed conversions, and increase in disease rate. Several studies of mycotoxicoses in domestic birds suggest the predisposal of birds ingesting mycotoxins to *Salmonella* infections (103, 108). Investigation of dietary T-2 toxin and mortality rates in chickens having paratyphoid infections revealed that neither factor alone causes extensive lethality, but a combination of the two significantly increases mortality (B. Boonchuvit, P. B. Hamilton, and H. R. Burmeister, in preparation). This work suggests that the inflammatory and irritant action of T-2 toxin toward the gastrointestinal tract reduces the natural barriers to invasion by *Salmonella*. Other studies demonstrated that aflatoxin-contaminated feed made birds more susceptible to low temperature, drinking water containing 1% NaCl, and certain disease symptoms associated with *Candida albicans* infection (36). Decreased resistance was attributed to a dramatic immunosuppression observed in birds consuming aflatoxin (114). Diseases related to aflatoxin toxicity have also been linked to interaction of the mycotoxin with viral infections (49, 110) and to a disorder of humans of unknown etiology called Reye's syndrome that is characterized by encephalopathy and fatty degeneration of viscera (7, 24).

HORMONAL MODIFICATION OF MYCOTOXIN EFFECTS

Zearalenone, or F-2, a product of several *Fusarium* spp., has estrogenic properties. A typical response of animals ingesting feed contaminated with F-2 involves primarily changes in the genital tract (60). Zearalenol, a derivative of zearalenone, is used under controlled conditions to promote beef cattle growth and as a therapeutic agent to alleviate postmenopausal discomfort (60). Zearelenone functions directly as a hormonal agent.

The interaction between aflatoxin and the hormonal system of test animals appears to be somewhat more subtle than the zearalenone effect. Preliminary interest in the aflatoxin effect on hormonal activity developed from consistent observations that male test animals were more susceptible than females to the toxicity of the mycotoxin (23, 69). Goodall and Butler (29) found that hypophysectomized rats did not develop liver tumors after ingestion of aflatoxin (4 ppm of B_1); lower levels of the toxin induced liver tumor formation in all control animals. Earlier, others had shown that hypophysectomization inhibited liver tumors elicited by azo dyes and aminofluorenes (30). Apparently, these liver carcinogens require metabolic activation before they interact

with the receptor site involved in the initial carcinogenic event(s).

Addition of diethylstilbestrol to an aflatoxin-contaminated ration also inhibited carcinogenesis in male rats (68). The incidence of liver carcinoma induced by aflatoxin was 71% compared with only 20% in animals ingesting the diet amended with the synthetic estrogen. Paired-feeding results showed that the difference between the occurrence of tumors could not be attributed to variations in feed intake.

Madhaven (52) sought the effect of the steroid prednisolone on aflatoxin toxicity in rats fed low-protein (5%) and high-protein (20%) rations. He observed no significant histological changes in livers of animals on the high-protein plus aflatoxin diet. Rats on the low-protein plus toxin ration developed typical liver lesions. However, in the presence of prednisolone, there was a marked inhibition of aflatoxin-induced bile duct proliferation and fat accumulation in the liver.

Cardeilhac and Nair (12) extended the study of endocrine involvement in aflatoxin-mediated carcinogenicity. The effect was determined in castrated male and female rats of a total dose of 2.15 mg of aflatoxin B_1. Acute toxicities were highest in uncastrated animals, and all survivors in the group had hepatomas 13 months after the test began. No liver tumors were observed in aflatoxin-treated, castrated rats or in the control animals that were not treated with toxin. This study also provided evidence that subcutaneous injection of CCl_4 increased the acute toxicity and carcinogenicity of aflatoxin.

Righter et al. (91) compared the effect of age and sexual status on susceptibility of rats to aflatoxin. In 12-week-old males after 2 weeks on a ration containing 12 ppm of aflatoxin, the mortality rate was 75 to 100%. However, prepubertal males (5 weeks old) ingesting the same ration had no signs of toxic stress. If castration was performed before 10 weeks of age, a 100% sparing effect was observed. Treatment of the castrated animals with testosterone restored sensitivity to aflatoxin.

MECHANISM OF MYCOTOXIN SYNERGISM

Many drugs, environmental chemicals, and endogenous cellular metabolites (e.g., steroid hormones, thyroxin, and bilirubin) are chemically modified by processes associated with microsomes located in the endoplasmic reticulum of liver cells (17, 18). One of the primary enzymatic reactions of the microsomes is a mixed function oxygenation (19, 40; Fig. 2).

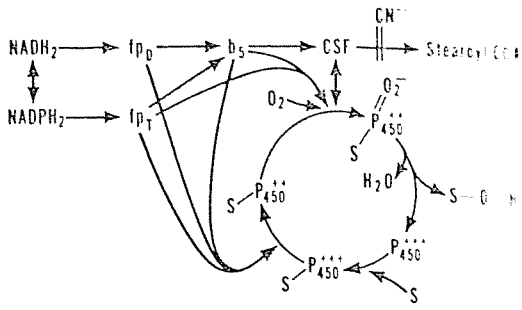

FIG. 2. *Electron transfer and cytochrome P (19, 40).*

Reducing equivalents for the reaction are provided primarily by reduced nicotinamide adenine dinucleotide phosphate, but reduced nicotinamide adenine dinucleotide can also provide electrons. Cytochrome P_{-450} serves as the terminal oxidase. The oxidized form of the hemoprotein (P_{-450}^{+++}) binds the substrate and undergoes a one-electron reduction. The reduced enzyme-substrate complex combines with molecular oxygen. Addition of a second electron leads to an oxidized substrate and water.

Current theory of chemical carcinogenesis requires that the carcinogen react with critical cellular molecules (59). Although no common structural feature is associated with all carcinogens, clearly, most of them are metabolized to reactive intermediates that are electrophilic; microsomal enzymes are responsible for this activation. The electrophilic intermediates combine with nucleophilic (electron rich) groups in proteins and nucleic acids through covalent bonds. Since neoplasms are characterized by an absence of growth control, apparently the ultimate carcinogen must bind to target macromolecules involved in the informational aspect of growth control before the carcinogenic process can be initiated.

It has been proposed that aromatic compounds are metabolized in higher organisms to phenols, dihydrodiols, and glutathione conjugates through intermediate formation of epoxides (arene oxides; 21, 41). The dihydrodiols may be either conjugated with glucuronic acid or converted, like catechol, to guaicol by the action of catechol-*O*-methyltransferase (Fig. 3). Epoxide hydration is mediated by an epoxide hydrase in liver microsomes; liver enzymes also have been identified that catalyze glutathione conjugation to the oxides (21). Products of microsomal processing have been isolated from urine of test animals; the substances appear to be harmless to the host. In addition to undergoing deactivation reactions,

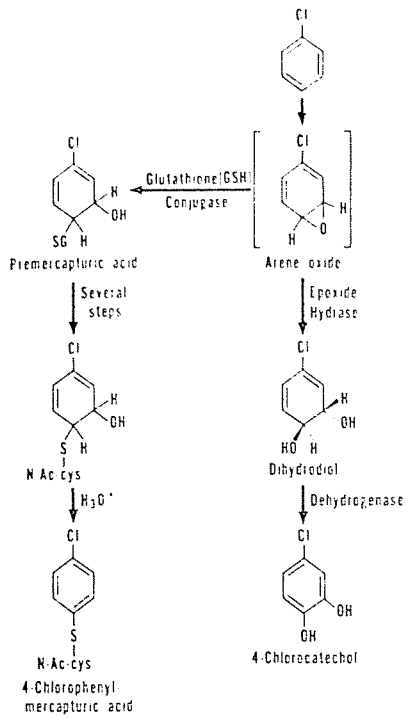

FIG. 3. *Processing of chlorobenzene in the liver (21, 41).*

the arene oxides and K-region epoxides are active electrophiles and readily form covalent bonds with cellular nucleophiles, such as proteins and nucleic acids. It has been proposed that the epoxides or arene oxides are the ultimate carcinogens (59).

Several hundred drugs, insecticides, carcinogens, steroid hormones, and other substances have been identified as stimulators or inhibitors of microsomal enzyme activity (18). Administration to rats of benzo(a)pyrene or other structurally similar, carcinogenic, polycyclic hydrocarbons increases microsomal hydroxylase activity. Enhanced metabolism of benzo(a)pyrene is reflected in increased biliary excretion of related products and a decreased level of the carcinogen in blood and other tissues (18). Inhibition of microsomal activity by carbon tetrachloride, β-diethyl-aminoethyl-propylacetate (SK 525-A), and piperonyl butoxide increases the toxicity of the polycyclic hydrocarbon (18, 21). Variation in microsomal activity can also have dramatic effects on the hormonal status of test animals since hydroxylation of steroids is mediated by the same system that metabolizes drugs and environmental chemicals.

In addition to the interaction between drugs or environmental chemicals and microsomal levels, other factors are involved. Microsomal enzyme induction is related to the nutrition of test animals. Protein deficiency reduces both microsomal protein and induction potential (11), whereas certain lipids in the diet govern the extent of microsomal induction (57). Carcinogenic synergism between cyclopropenoid fatty acids (CPFA) and aflatoxin may also reflect an interaction between the substances in microsomal metabolism; for example, CPFA inhibit the microsomal-mediated desaturation of stearic to oleic acid (43).

Increase of microsomal enzymes requires deoxyribonucleic acid (DNA)-dependent RNA synthesis and protein production. Substances that modify transcription, translation, or aggregation could change the actual number of functioning microsomes (17, 18). In addition, the rates of microsomal enzyme activity can also be modified by certain compounds without quantitative increase in enzyme concentration (11); the change resembles allosteric alteration. Marshall and McLean (57) have suggested that an endogenous cellular factor normally inactivated by microsomes serves as the inducing agent for synthesis of microsomal components. When other substrates compete with the factor for common enzyme sites on the microsomes, levels of the inducer increase and the induction process increases. A satisfying model of microsomal regulation can be developed with steroid hormones as endogenous inducing factors; the substances could effectively balance basal induction of microsomes with steady-state control based on competition with other compounds in the cellular milieu for active sites on the catalytic complex (Fig. 4).

In vivo microsomal activity is difficult to predict; it is questionable whether the net

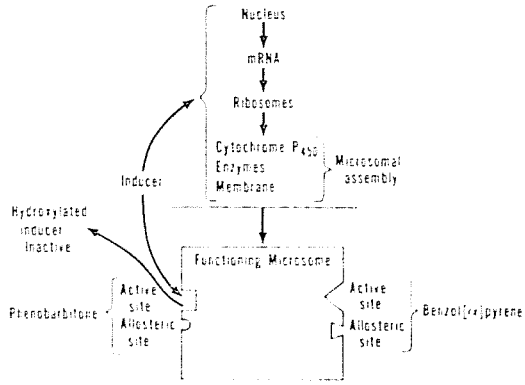

FIG. 4. *Proposed mechanism for microsomal induction.*

effect of an increased rate of metabolism either will produce a decrease in toxicity via enhanced destruction of the toxin or will augment toxicity caused by an increase in the formation of the active intermediate(s). Several factors influence the net toxicity of a substance: (i) rate of absorption from the intestinal tract into the blood stream, (ii) transfer rate from blood into cells of specific tissues, (iii) kinetics of binding to microsomes and conversion rate, (iv) binding rate of toxic intermediate to critical target molecules, and (v) rate of toxicity expression of the toxic intermediate-target molecule complex (28; Fig. 5).

In early studies with aflatoxin B_1 metabolism in test animals, a fluorescent hydroxylated metabolite, aflatoxin M_1, was found in milk, urine, and feces (23, 46). Structural characterization showed that M_1 was hydroxylated on the bridgehead carbon of the bifuran (23). Conjugates of M_1 were observed in urine and bile of test animals (5). Further studies (72, 73, 81, 98) verified that liver microsomes from several species were capable of converting B_1 to M_1. Subsequent reports have described additional microsomal hydroxylations of B_1 (Fig. 6): (i) *O*-demethylation to a phenolic derivative—P_1 (20), (ii) hydration of the vinyl ether double bond of the furan to yield the 2-hydroxy derivative—B_{2a} (73, 99), and (iii) addition of a hydroxyl group to the carbon β to the carbonyl on the cyclopentenone ring—Q_1 (58). Patterson and Roberts (74) have identified a soluble liver enzyme that reduces the carbonyl on the cyclopentenone to an alcohol, aflatoxicol (22).

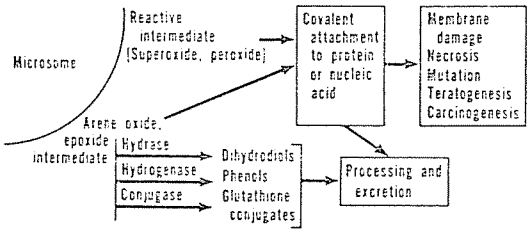

FIG. 5. *Proposed mechanisms for conversion of reactive microsomal intermediates.*

Induction of microsomal enzyme activity by phenobarbitone and DDT reduces the toxicity of aflatoxin in rats (31, 50, 51). Schabort and Steyn (98) observed that livers of rats dosed with either phenobarbital or aflatoxin B_1 exhibited microsomal aflatoxin-4-hydroxylase (M_1) activity 2.3 and 3.5 times higher than controls. On the other hand, Gurtoo and Campbell (33) found that 3,4-benzpyrene induced microsomal enzyme synthesis but that aflatoxin did not; both compounds increased the enzyme activity in situ. They attributed the absence of microsomal enzyme synthesis in the presence of aflatoxin to inhibition of transcription by the toxin. The apparent discrepancy in observations is reminiscent of the condition described by Pollack (80) in actinomycin D-mediated induction of penicillinase synthesis in *Bacillus licheniformis*. The observed stimulation of the inducible enxyme by low levels of actinomycin D, a transcription inhibitor, was attributed to a preferential binding of the compound to the regulator gene and release of the structural gene from repression. When regulator genes were saturated, increased quantities of actinomycin associated with structural genes and blocked penicillinase production. The analogy between the activity

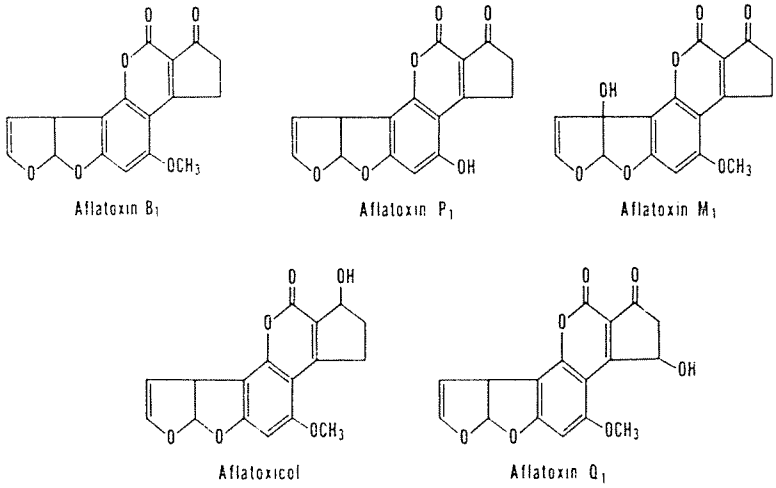

FIG. 6. *Structures of aflatoxin B_1 and derivatives.*

of actinomycin D and aflatoxin is appropriate since the function of the two substances is quite similar, particularly in inhibition of transcription (23).

Schoental (101) proposed that the reactive intermediate of aflatoxin B_1 was an epoxide formed across the 2,3 double bond of the terminal furan. Garner (26) and others (112) provided evidence for the 2,3 epoxy aflatoxin B_1 in microsomal conversion of the toxin and showed that liver preparations catalyzed binding of B_1 of RNA in vitro (Fig. 7). The double bond of the B_1 furan appeared to be an important structural characteristic for epoxide formation since aflatoxin B_{2a}, the 2,3 dihydro derivative, did not bind to nucleic acid in the microsomal activation test. In a perceptive study of the effect of liver microsome preparations plus aflatoxin B_1 incubated in the presence of viable cells of *Salmonella*, Garner et al. (27) found that cells were killed rapidly: 99% of the cells were dead after a 2-min incubation. This lethality was attributed to the active intermediates(s) of B_1 metabolism. Subsequently, Ames et al. (2) developed a mutagen test system using liver microsomes and a set of *Salmonella* strains. Microsomal-activated intermediates of aflatoxin B_1, sterigmatocystin, and other carcinogens were all mutagens (2).

The actual binding of tritiated aflatoxin B_1 to macromolecules in vivo was explored by

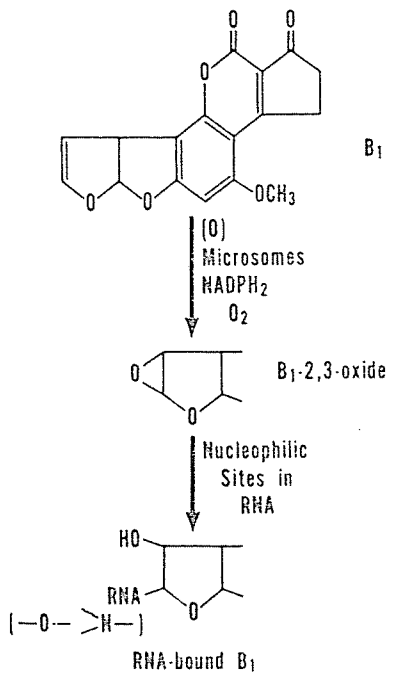

FIG. 7. *Proposed mechanism of binding of aflatoxin B_1 epoxide intermediates to RNA (26, 112).*

Lijinsky et al. (45). Maximal incorporation of radioactivity in DNA, RNA, and protein was observed in liver from 6 to 18 h after toxin administration. The isotope was detected in cellular constituents up to 8 weeks after treatment. There was no apparent correlation between binding by macromolecules and the carcinogenic process. The influence of microsomal processing of aflatoxin on informational enzymes has also been evaluated. Activity of deoxyribonucleases of rat and mouse liver increased after in vivo treatment with aflatoxin (77, 78). On the other hand, both in vivo and microsome-containing in vitro systems with aflatoxin B_1 inhibited certain RNA polymerase activities (1, 62, 95).

Further studies of species differences in aflatoxin metabolism led to the discovery in avian and rabbit liver of an enzymatic function that reduced aflatoxin B_1 to aflatoxicol (72, 74). Since the reduction was reversible, Patterson and Roberts (72) proposed that the B_1-aflatoxicol reaction provided for an aflatoxin reservoir within liver cells before microsomal processing. These workers postulated a slightly different mechanism of microsomal activation of B_1; they suggested that the hemiacetal, aflatoxin B_{2a}, is the toxic intermediate (73, 74). Their provocative proposal is based on the reactivity of the dialdehyde form of B_{2a} particularly in forming Schiff bases with free amino groups of proteins (Fig. 8). Schabort and Pitout (97) also provided evidence for covalent binding of B_{2a} to proteins. Since the hemiacetal is unstable at physiological pH (79), it may represent an intracellular reactive intermediate. Gurtoo (32) and Gurtoo and Dahms (34) observed that a microsomally mediated intermediate of aflatoxin B_1, provably B_{2a}, binds covalently to microsomal protein.

While looking at the cytoplasmic reduction of B_1 to aflatoxicol, Patterson and Roberts (75) found that 17-ketosteroid sex hormones inhibit the reaction; such an interaction could be an important aspect of the cellular response to aflatoxin (Fig. 9). Williams et al. (118, 119) studied the relationship between aflatoxin binding and ribosome-membrane association. They observed an aflatoxin-mediated detachment of ribosomes from rough endoplasmic reticulum. The attachment site of the toxin was identified as the steroid-dependent ribosome binding site. Aflatoxin inhibited the ability of steroids to promote polysome binding to endoplasmic reticulum membranes.

The enhanced resistance of female rats to aflatoxin has been attributed to an increased

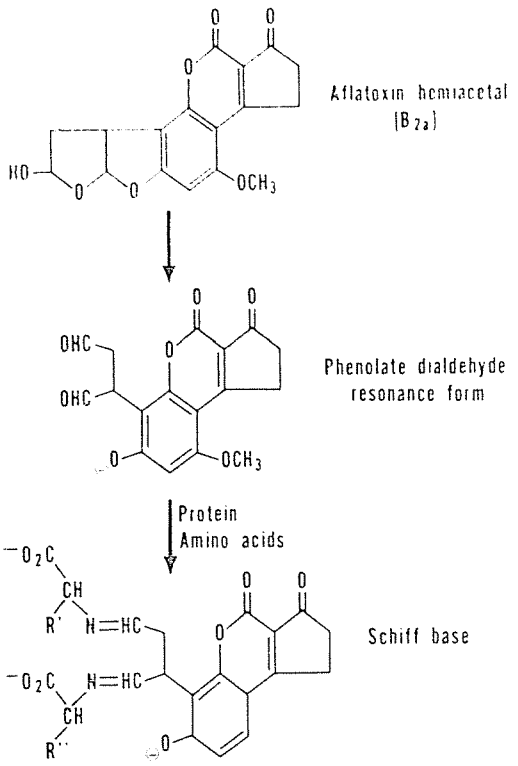

FIG. 8. *Conversion of aflatoxin B_{2a} to Schiff base.*

rate of microsomal conversion of B_1 (82). Although M_1 is only one of several metabolic products of B_1, tissue levels of the 4-OH derivative have been studied as an indicator of the rate of B_1 detoxification (73, 82, 84). Higher levels of M_1 have been observed in organs of female rats than in similar tissues of males; the sex difference is eliminated by castration (83). However, administration of specific hormones to animals did not significantly change tissue levels of M_1. Purchase and Steyn (83) suggested that hormonal effects

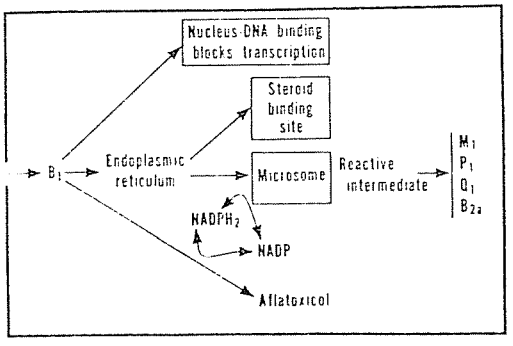

FIG. 9. *Proposed mechanisms of aflatoxin B_1 reaction in liver cells (75).*

may be nonspecific; i.e., increased microsomal activity in female livers may represent inherently higher endogenous levels of steroids and attendant microsomal activity in the female rats than in males.

Although cellular processes involved in expression of mycotoxin synergisms are complex, the growing understanding about reactions associated with microsomal metabolism will provide the fundamental ingredients for a future comprehensive explanation. The response of biological systems to mycotoxins, coupled with the larger spectrum of environmental chemicals, is a profitable area of inquiry. Hopefully, both vitality and quality of current work will be sustained.

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